Bacterial Persistence as a Phenotypic Switch

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cardia bifida. Mutations in several zebrafish genes expressed in the foregut endoderm, including *casanova* (*sox32*) and *faust* (*GATA5*), result in cardia bifida, and these mutants display severe defects or complete lack of foregut endoderm development (*22*, *23*). In mice lacking *GATA4*, anterior foregut endoderm development is defective, and *GATA4*-null embryos display cardia bifida (*3*, *4*). Thus, the correlation between foregut defects and cardia bifida phenotypes is very strong. However, there are distinct differences between these mutants and *Foxp4* mutants. *GATA4* mutants have severe defects in ventral morphogenesis and lack proper cardiac chamber development (*3*, *4*). Moreover, zebrafish cardiac morphogenesis is distinctly different from that in mammals, in that there are only two chambers and the heart does not loop or septate, but instead forms a serial connection between the single atria and ventricle. Although other models of cardia bifida have been reported, including *Mesp1* and *furin* mutant mice, these embryos either die too early to examine the complex morphogenetic processes required for late-stage cardiac development or they exhibit other severe defects in general developmental processes such as embryonic turning (*5*, *6*, *11*). Thus, none of the cardia bifida mutants described previously has permitted the analysis of complex cardiac development in sufficient detail to determine whether the later stages of cardiac morphogenesis require heart tube fusion.

Foxp4 mutant embryos demonstrate that bilateral heart tube migration and fusion are not required for extensive cardiac development, including chamber formation, ventricular myocyte differentiation, looping, endocardial cushion formation, and development of cardiac left-right asymmetry. These data indicate a higher degree of preprogramming in the bilateral precardiac mesoderm than has been appreciated and indicate that a redefinition of the current model of cardiac development is required (*1*).

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S4
Table S1
References and Notes

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Bacterial Persistence as a Phenotypic Switch

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A fraction of a genetically homogeneous microbial population may survive exposure to stress such as antibiotic treatment. Unlike resistant mutants, cells regrown from such persistent bacteria remain sensitive to the antibiotic. We investigated the persistence of single cells of *Escherichia coli* with the use of microfluidic devices. Persistence was linked to preexisting heterogeneity in bacterial populations because phenotypic switching occurred between normally growing cells and persister cells having reduced growth rates. Quantitative measurements led to a simple mathematical description of the persistence switch. Inherent heterogeneity of bacterial populations may be important in adaptation to fluctuating environments and in the persistence of bacterial infections.

When a population of genetically identical bacterial cells is exposed to a sufficiently strong antibiotic treatment, most of the population is killed. Killing can be measured by monitoring the fraction of viable cells as a function of the exposure to the antibiotic treatment. The resulting killing curve for wild-type *Escherichia coli* is plotted in Fig. 1A. The death of the majority of the population takes place as a fast exponential decay characterized by a single parameter, the killing rate. After a few hours, the initial purely exponential decay of the killing curve changes to a more complex, slowly decreasing function. By the time the antibiotic is removed, a small fraction of the cells still survives. These cells have not genetically acquired antibiotic resistance: They regrow a new population that is as sensitive to the antibiotic. This phenomenon, termed bacterial persistence, was first reported for staphylococcal infections treated with penicillin (*1*) and has since been observed in many bacterial species. Despite being observed almost 60 years ago, the mechanism behind persistence remains a puzzle (*2*). It has been suggested that persistent bacteria are in some protected part of the cell cycle at the time of exposure to antibiotics or are able to adapt rapidly to the antibiotic stress (*3*). It has also been proposed that those cells are in a dormant state or are unable to initiate programmed cell death (*2*, *4*). To clarify the nature of persistence, it is crucial to know whether persistent bacteria differ from others before exposure to antibiotics and, if so, what triggers such phenotypic differentiation.

To answer these questions quantitatively, we investigated the persistence of *E. coli* at the level of single cells by direct observation and measurement with the use of optical microscopy. Such measurements were made possible by the use of previously isolated high persistence (*hip*) mutants of *E. coli* that have an increased proportion of persisters (*5*, *6*) and by the recent development of transparent microfluidic devices (*7*, *8*).

We designed and fabricated microfluidic devices by using the techniques of soft lithography (*9*, *10*) to pattern layers of poly (dimethylsiloxane) (PDMS) (Fig. 1B). With

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these devices, we can record the growth of individual bacteria under normal conditions, expose them to antibiotic treatment, detect the rare survivors, and analyze the survivors' history. Even before the antibiotic treatment, all the observed persisters could be clearly distinguished from the normal cells by their reduced growth rate. These single-cell observations allowed us to describe mathematically the switching behavior between rapidly growing normal cells, *n*, and nongrowing or slowly growing persister cells, *p*, in the framework of a simple two-state model (Fig. 2). Once the mechanism of persistence was demonstrated at the level of single cells, measurements of the parameters of the model could be done in batch cultures. The growth-death rates of the persisters and the normally growing cells were characterized in this model by the constants μ_p and μ_n, respectively. The cells switched from the *n* state to the *p* state with a constant rate *a* or from the *p* state to the *n* state with a constant rate *b*.

We first chose to study *hip* mutants with the *hipA7* allele, isolated in a pioneering work by Moyed and colleagues (*5*). Interestingly, the persistence of the *hipA7* allele after ampicillin exposure was shown to be linked to persistence in many other stresses (*6*, *11*). The killing curve of these mutant cells (Fig. 1A) is well described by double-exponential kinetics: The majority of the population is characterized by a fast killing time (25 min), whereas the subpopulation of persisters dies off over a much longer characteristic time (6 hours) (*12*). A good fit to experimental data is obtained by the two-state model presented in Fig. 2.

For single-cell measurements, the *hipA7* bacteria are first grown in a microfluidic device under the microscope on Luria-Bertani Lennox medium (LBL) (Fig. 1C) (*12*). During growth, the descendants of each bacterium form a separate linear microcolony (Fig. 1, C to E). By using time-lapse microscopy and measuring the length of newly formed linear microcolonies, we derived growth rates of the progeny of individual cells. The average growth rate of *E. coli* in our devices was the same as that for batch cultures. After several cell divisions, ampicillin was added to the medium, and the death of cells, accompanied by lysis, was easily observed (Fig. 1F). After 5 hours of ampicillin, killing slowed, and ampicillin was cleared from the device with fresh LBL (Fig. 1G). After the removal of ampicillin, rare bacteria, which survived, started growing and dividing again (Fig. 1, G and H): These were identified as the persister cells. The persistence phenotype was not due to spatial inhomogeneities inside our devices: The locations of persisters showed no pattern, and these cells were often found in close proximity to nonpersister cells. Following the behavior of those cells back in time (movie S1) (*12*), we observed that they differed in their growth rate from the majority of the population before the exposure to antibiotics. Persister cells, which seemed to be in an arrested growth state, could spontaneously switch to fast growth and generate a population that is sensitive to the antibiotic (*12*) (fig. S1). We thus conclude that persistence in the *hipA7* population is linked to an inherent heterogeneity of growth rates in the bacterial population.

We have established the following properties of *hipA7* persisters, characteristic of what we call type I persisters:

1) Type I persisters constitute a preexisting population of nongrowing ($\mu_p \approx 0$) cells that are generated at stationary phase.

2) Type I persistence is characterized by a negligible spontaneous switching rate from *n* to *p* during exponential growth ($a \approx 0$) (*13*). In batch culture (Fig. 3B), the number of persister cells is directly proportional to the number of stationary phase cells inoculated into the culture, consistent with recent observations (*14*, *15*) .

3) Type I persisters inoculated into fresh medium from stationary phase switch back to growing cells with a characteristic extended time lag. The *hipA7* population consists of two distinct subpopulations, each characterized by a different time constant for the exit from stationary phase (Fig. 3A). The apparent lag time for the persister population is the inverse of *b*, the switching rate from *p* to *n*. We measured *b* by plating an overnight culture of the *hipA7* mutant on LBL agar plates and monitoring the appear-

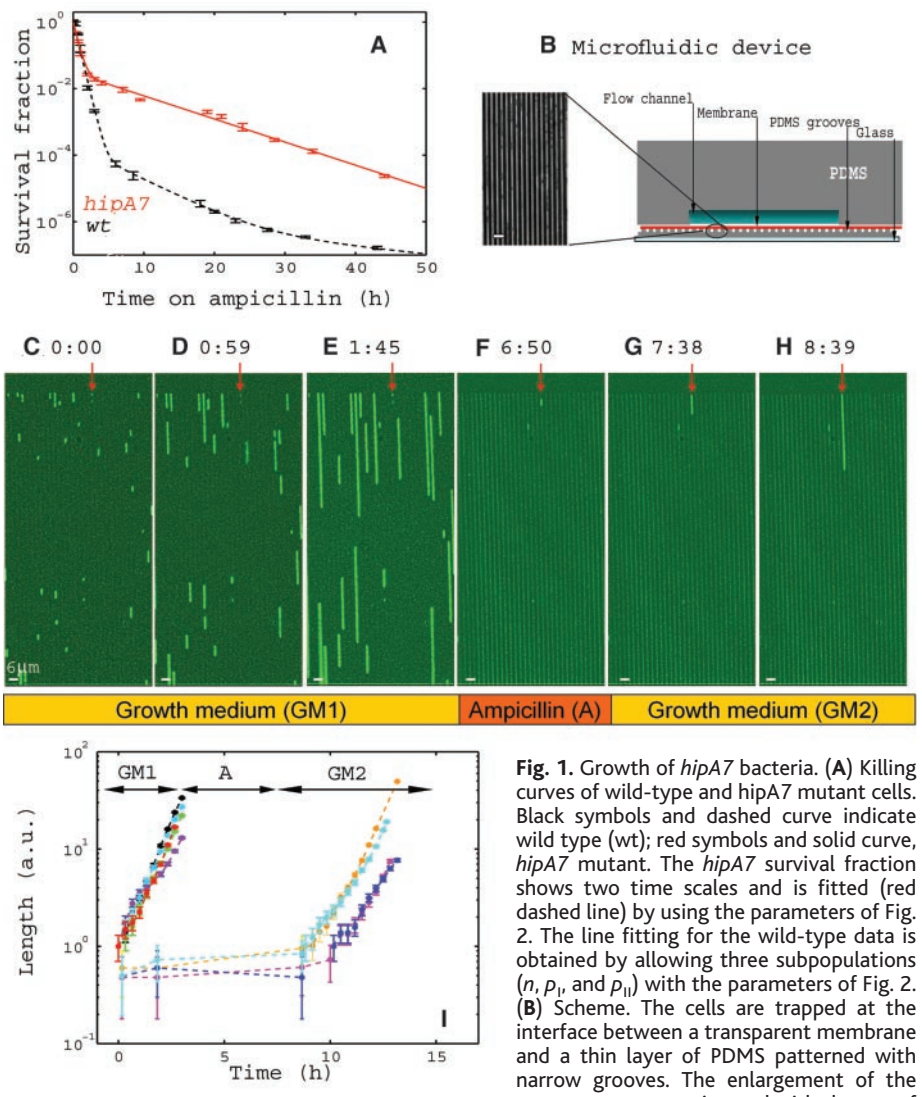


Fig. 1. Growth of *hipA7* bacteria. (**A**) Killing curves of wild-type and hipA7 mutant cells. Black symbols and dashed curve indicate wild type (wt); red symbols and solid curve, *hipA7* mutant. The *hipA7* survival fraction shows two time scales and is fitted (red dashed line) by using the parameters of Fig. 2. The line fitting for the wild-type data is obtained by allowing three subpopulations (n, p_I, and p_{II}) with the parameters of Fig. 2. (**B**) Scheme. The cells are trapped at the interface between a transparent membrane and a thin layer of PDMS patterned with narrow grooves. The enlargement of the groove pattern was imaged with the use of phase-contrast microscopy. (**C** to **H**) Time lapse of *hipA7* cells expressing yellow fluorescent protein. Times from $t = 0$ are indicated in hours:min. (C to E) Bacteria taken from an overnight culture are exposed to growth medium (GM1) and divide on narrow grooves, thus forming strings of cells originating from the same cell. (F) Same field of view after exposing the cells to ampicillin (denoted by A). Only persister cells remain. (G and H) Same field of view after removal of the ampicillin by washing with growth medium (GM2). The red arrow points to the location of a type I persister bacterium. (**I**) The length of the lineages of several *hipA7* cells is monitored during GM1, A, and GM2 time periods. Individual cells are plotted as different colors. Type I persister cells do not grow during the GM1 and A periods. a.u., arbitrary units.

ance of visible colonies (Fig. 3A) (*12*). The majority of the population is characterized by a short lag time (40 min) comparable to the wild-type lag, whereas a subset is characterized by a lag time of about 14 hours (b = 0.07 hours^{-1}). Upon introduction of fresh nutrients, cells with the shorter lag time rapidly begin growth and become susceptible to killing by ampicillin, whereas the rest remain dormant and less sensitive to ampicillin, which is known to target mainly growing cells (*16*, *17*).

The fraction of persisters in the wild-type population is more than three orders of magnitude smaller than in the *hipA7* population, making their detection under the microscope or by a colony appearance assay on plates very difficult. Despite similarities in the initial behavior of the wild-type and the *hipA7* data, the killing curve for the wild type is more complex at longer times and cannot be fitted by using only two time constants (Fig. 1A). In contrast to the *hipA7* persisters, whose number is independent of the total number of growing cells, the number of wild-type persisters increases as the total population increases (Fig. 3B). We conclude that type I persistence does not fully characterize wild-type persisters.

Another mutant, *hipQ*, was previously isolated in a screen for high persistence to norfloxacin treatment and, like *hipA7*, was later found to persist through treatment with several antibiotics, including ampicillin (*6*). However, the *hipQ* locus is found in a different region of the chromosome and has not been fully characterized. Our experiments show that the *hipQ* persisters can be described within the same mathematical model (Fig. 2) but with dynamics that differ in important ways from type I. These persisters, which we call type II persisters, constitute a subpopulation of slowly growing cells ($\mu_p \neq 0$). An inherent heterogeneity in the growth rate was also present in the *hipQ* mutants before the antibiotic treatment (Fig. 4) (*18*). In contrast to *hipA7* persisters, which are found in an arrested growth state, *hipQ* persisters grow and divide continuously but an order of magnitude slower than nonpersisters (Fig. 4G and movie S2). This persistence growth state is inherited for several generations (fig. S2).

Type II persisters do not appear to originate from passage through stationary phase. The number of persisters in a growing culture is determined by the total number of cells and not by the size of the inoculum from stationary phase ($a \neq 0$) (table S1). The dynamics of type II persistence for the *hipQ* population qualitatively follows the equations presented in Fig. 2. Batch culture experiments (Fig. 4H) showing the ability of *hipQ* persister cells to switch back to fast growth allowed us to set a lower limit on b (Fig. 2).

We now turn to the more complex wild-type population dynamics. Persisters in the wild-type population are continuously generated during exponential growth (Fig. 3B) ($a \neq 0$). However, passage through stationary phase also increases the number of persisters (table S1). The wild-type population can be thus described as consisting of three subpopulations: normal cells, n; continuously generated type II persisters, p_{II}; and stationary phase type I persisters, p_I. The dynamical model can then be used to fit the killing curve of the wild-type population (Fig. 1A) and

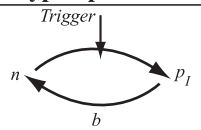
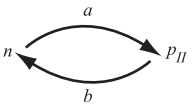
Type I persisters		Type II persisters	
			
$\begin{cases} \frac{dp_I}{dt} = -bp_I + \mu_p p_I \\ \frac{dn}{dt} = bp_I + \mu_n n \end{cases}$ Eq.(1)		$\begin{cases} \frac{dn}{dt} = -an + bp_{II} + \mu_n n \\ \frac{dp_{II}}{dt} = an - bp_{II} + \mu_p p_{II} \end{cases}$ Eq. (2)	
Estimated rates (units: hours^{-1})			
hipA7: b=0.07±0.01	*wt* Type I subpopulation: μ_p-b= -0.05±0.01*(23)*	*wt* Type II subpopulation: a=1.2±0.2 x10^{-6} b=0.1±0.05	*hipQ* a=1.0±0.2x10^{-3} 10^{-7}<b<10^{-4}
For a general solution of Eqs (1&2) see the Supplementary Online Material			

Fig. 2. Mathematical description of persistence. General equations describing the dynamics of two subpopulations, here denoted as normal (n) and persister (p) cells. Type I persisters are generated by trigger events during stationary phase, whereas type II persisters are continuously generated during growth. The parameters for the *hip* populations and for the wild type are determined by fitting the plots of Figs. 1, 3, and 4 to the solutions of the kinetics equations (Eqs. 1 and 2).

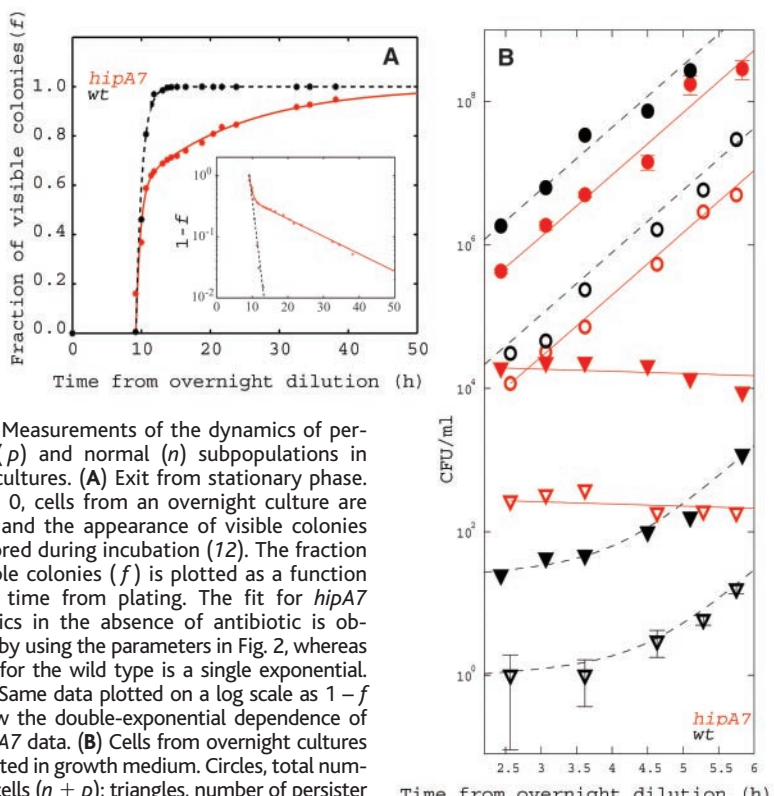


Fig. 3. Measurements of the dynamics of persister (p) and normal (n) subpopulations in batch cultures. (**A**) Exit from stationary phase. At t = 0, cells from an overnight culture are plated and the appearance of visible colonies monitored during incubation (*12*). The fraction of visible colonies (f) is plotted as a function of the time from plating. The fit for *hipA7* dynamics in the absence of antibiotic is obtained by using the parameters in Fig. 2, whereas the fit for the wild type is a single exponential. (Inset) Same data plotted on a log scale as $1 - f$ to show the double-exponential dependence of the *hipA7* data. (**B**) Cells from overnight cultures are diluted in growth medium. Circles, total number of cells ($n + p$); triangles, number of persister cells (p); open and solid symbols, dilutions from overnight cultures of 10^{-6} and 5×10^{-5}, respectively. Fits represent the solutions of Eqs. 1 and 2 with the parameters of Fig. 2. During exponential growth of the total population, the number of persisters decreases slowly for the *hipA7* mutant, whereas it increases for the wild type.

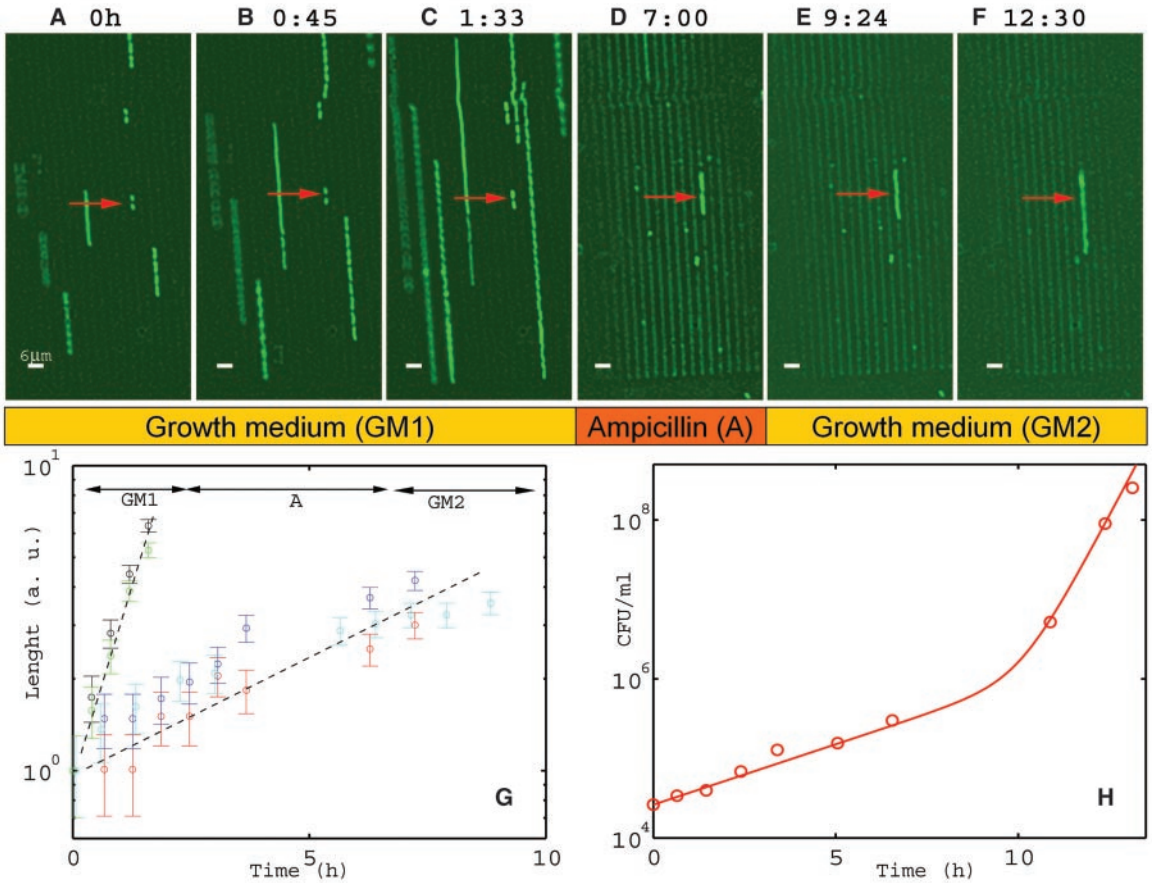


Fig. 4. (**A** to **F**) Growth of *hipQ* bacteria in a microfluidic chamber. Time lapse of *hipQ* cells expressing yellow fluorescent protein. (A to C) Bacteria taken from an exponentially growing culture are exposed to growth medium (GM1) and divide on narrow grooves, thus forming strings of cells originating from the same cell. (D) Same field of view after exposing the cells to 4 hours of ampicillin (denoted by A). Only few cells remain. (E and F) Same field of view after removal of the ampicillin by washing with growth medium (GM2). The red arrow points to the location of a type II persister bacterium. (**G**) Growth of *hipQ* bacteria in a microfluidic chamber. The length of the lineages of several cells are monitored during GM1, A, and GM2. The cells that grow quickly during GM1 die during A. The type II persister cells grow slowly during GM1, A, and GM2. (**H**) Switch of *hipQ* persisters in batch cultures from slow growth to fast growth after an antibiotic treatment of 4.5 hours. The red line was obtained by fitting the solutions of Eqs. 1 and 2 with free parameter b, whereas μ_p and μ_n were determined from the growth rates near $t = 0$ and $t \gg 0$, respectively, and a was determined from the measurement of the persisters ratio (table S1). CFU, colony-forming units.

extract the different time scales characterizing the three subpopulations (Fig. 2). Despite the good agreement between the simple theoretical model and the experimental data, we cannot exclude the possibility of other persister types.

Possibly, persisters have been selected to increase chances of survival of bacterial populations in fluctuating environments (fig. S3). Similar strategies are not uncommon in more complex organisms: Variable maturation rates in insects or germination events in plant seeds have been described as bet-hedging strategy for facing unpredictable environments (*19*). The quantitative characterization of persistence states and the associated phenotypic transitions should find clinical application in treatment of pathogens such as *Mycobacterium tuberculosis* (*20*), *Staphylococcus aureus* (*3*), and *Pseudomonas aeruginosa* in cystic fibrosis patients (*21*). The identification of the switch responsible for persistence suggests different possible drug targets: Stationary phase–induced persistence could be reduced with factors that affect the lag period (*22*), whereas spontaneous persistence could be targeted by factors specifically enhancing the switching rate from persister to normal cells.

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12. Materials and methods are available as supporting material on *Science* Online.
13. The $a = 0$ approximation is valid in experiments where the amount of persistence of *hipA7* cells is high, that is, after passage through stationary phase, thus occluding detection of any substantially smaller subpopulations.
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18. We showed that the heterogeneity in the *hip* populations is not due to the ampicillin. However, the ability of each subpopulation to persist might be due to a response to the antibiotic itself.
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23. The small fraction of the type I persisters in the wild type does not allow the separation of the two variables.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S3
Table S1
Movies S1 and S2

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